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Abaloparatide Maintains Normal Rat Blood Calcium Level in Part Via 1,25-Dihydroxyvitamin D/osteocalcin Signaling Pathway

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Abstract

The PTH-related peptide(1-34) analog, abaloparatide (ABL), is the second anabolic drug available for the treatment of osteoporosis. Previous research demonstrated that ABL had a potent anabolic effect but caused hypercalcemia at a significantly lower rate. However, the mechanism by which ABL maintains the stability of blood calcium levels remains poorly understood. Our *in vivo* data showed that ABL treatment (40 µg/kg/day for 7 days) significantly increased rat blood level of 1,25-dihydroxyvitamin D [1,25-(OH)₂D] without raising the blood calcium value. ABL also significantly augmented the carboxylated osteocalcin (Gla-Ocn) in the blood and bone that is synthesized by osteoblasts, and increased noncarboxylated Ocn, which is released from the bone matrix to the circulation because of osteoclast activation. The *in vitro* data showed that ABL (10 nM for 24 hours) had little direct effects on 1,25-(OH)₂D synthesis and Gla-Ocn formation in nonrenal cells (rat osteoblast-like cells). However, ABL significantly promoted both 1,25-(OH)₂D and Gla-Ocn formation when 25-hydroxyvitamin D, the substrate of 1α-hydroxylase, was added to the cells. Thus, the increased 1,25-(OH)₂D levels in rats treated by ABL result in high levels of Gla-Ocn and transient calcium increase in the circulation. Gla-Ocn then mediates calcium ions in the extracellular fluid at bone sites to bind to hydroxyapatite at bone surfaces. This regulation by Gla-Ocn at least, in part, maintains the stability of blood calcium levels during ABL treatment. We conclude that the signaling pathway of ABL/1,25-(OH)₂D/Gla-Ocn contributes to calcium homeostasis and may help understand the mechanism of ABL for osteoporosis therapy.

Key Words: abaloparatide, osteocalcin, carboxylation, 1,25-dihydroxyvitamin D, blood calcium, bone biomarker

Abbreviations: 1,25-(OH)₂D, 1,25-dihydroxyvitamin D; 24,25-(OH)₂D, 24,25-dihydroxyvitamin D; 25-(OH)D, 25-hydroxyvitamin D; ABL, abaloparatide; Gla, gamma-carboxyglutamic acid; Glu, glutamic acid; Ocn, osteocalcin; PTHR, type 1 PTH receptor; PTHrP, PTH-related protein.

Many osteoporotic patients have already lost a substantial amount of bone at the time of diagnosis, and anabolic treatments for osteoporotic patients are needed to increase new bone formation in these patients. The recombinant human parathyroid hormone(1-34) (teriparatide) was approved as the first anabolic drug for the treatment of osteoporosis in the United States. The synthetic human PTH-related peptide(1-34) analog (abaloparatide [ABL]) as a second anabolic drug was approved in the United States for osteoporosis therapy in an attempt to improve its anabolic actions while mitigating the catabolic effects on bone (1, 2). Previous research from both animals and humans demonstrated that ABL had a potent anabolic effect but caused hypercalcemia at a significantly lower rate compared with teriparatide (3–5). The optimal use of ABL therapy in osteoporosis depends on our understanding of ABL signaling in both anabolic and catabolic effects. The bone is a metabolically active organ that undergoes continuous remodeling through the concerted actions of osteoblastic bone formation and osteoclastic bone resorption (6, 7). Osteocalcin (Ocn), whose name is related

with calcium and its location in the bone, contains 3 gamma-carboxyglutamic acid (Gla) residues and is the most abundant noncollagenous protein in the bone (8, 9). Posttranslational modification in osteoblasts results in the majority of Ocn being completely carboxylated. The carboxylated Ocn (Gla-Ocn), which has the 2 carboxyl groups (10), is released into the bone matrix and binds to hydroxyapatite at bone surfaces to elevate the saturation point for the solubility of hydroxyapatite. The calcium ions in the extracellular fluid at bone sites are then able to move to the bone mineral surface because of bone surface areas in contact with the extracellular fluid, thereby playing a role in calcium homeostasis (11, 12). Thus, Gla-Ocn is mostly incorporated into bone matrix and some levels of Gla-Ocn in the circulation reflect osteoblast function. During bone remodeling, Gla-Ocn in the bone matrix can be converted into a form with a lower grade of carboxylation and uncarboxylated Ocn (Glu-Ocn), and then be released into the bloodstream when osteoclasts are activated to form an acidic environment (8, 9, 13). In contrast to Gla-Ocn, Glu-Ocn has no binding affinity to ionized calcium.

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The circulating levels of Glu-Ocn are therefore dependent on the rate of bone turnover. Both forms of Gla-Ocn and Glu-Ocn exist in the circulation and the ratio of Gla-Ocn to Glu-Ocn may mirror the status of bone remodeling after drug treatment (14, 15). Although ABL was reported to increase total Ocn formation in the circulation (5), the effects of ABL on both Gla-Ocn and Glu-Ocn levels and their ratio in the bloodstream and bone are currently unknown.

The effects of ABL are mediated by type 1 PTH receptor (PTHrR), which is primarily located in kidney and bone (16, 17). The synthesis of 1,25-dihydroxyvitamin D [1,25-(OH)₂D], the hormonally active form of vitamin D, is stimulated by PTHrR activation in the kidney and plays an important role in intestinal calcium absorption and renal calcium reabsorption (18, 19). It is known that PTH in primary hyperparathyroidism and PTH-related protein (PTHrP) in humoral hypercalcemia of malignancy have diverse effects on 1,25-(OH)₂D formation. In contrast with those with primary hyperparathyroidism, patients with humoral hypercalcemia of malignancy display marked reductions in circulating 1,25-(OH)₂D, indicating PTH but not PTHrP is able to induce 1,25-(OH)₂D formation (20-22). Recently, it was reported that ABL treatment could increase serum 1,25-(OH)₂D levels in osteoporotic patients (23). In addition, nonrenal cells, such as osteoblasts, express 1 α -hydroxylase, also produce 1,25-(OH)₂D (24). However, the effect of ABL on 1,25-(OH)₂D formation in bone cells has not been explored yet. The present study shows that ABL administration significantly increases 1,25-(OH)₂D synthesis without raising the calcium value in the circulation and stimulates the Gla-Ocn formation and high ratio of Gla-Ocn to Glu-Ocn in the blood and bone in rats.

Materials and Methods

Materials

ABL ([Glu^{22,25}, Leu^{23,28,31}, Aib²⁹, Lys^{26,30}] PTHrP [1-34]), in which Aib is an aminoisobutyric acid, was synthesized by PEPTIDE 2.0 (Chantilly, VA) (25). Rat Gla-Ocn highly sensitive EIA kit (Catalog No. MK126, RRID: AB_2940963) and rat Glu-Ocn highly sensitive EIA kit Glu-Ocn (Catalog No. 146, RRID: AB_2940964) were purchased from Takara Bio USA, Inc (San Francisco, CA). 1,25-(OH)₂D EIA kit (Catalog No. AC-62F1, RRID: AB_2891249) and 25-hydroxyvitamin D [25-(OH)D] EIA kit (Catalog No. AC-57SF1, RRID: AB_2756867) were purchased from Immunodiagnostic Systems Inc. Calcium colorimetric assay kits (Catalog No. ab102505) was purchased from Abcam. Cyclic AMP Direct EIA Kit (Catalog No. K019-H5, RRID: AB_2940962) was purchased from Arbor Assays (Ann Arbor, MI). 1,25-(OH)₂D (Catalog No. D1530), 25-(OH)D (Catalog No. H-083), vitamin K1 (Catalog No. V3501), and proteinase inhibitor cocktail (Catalog No. P8849) were purchased from Sigma-Aldrich (St. Louis, MO). 24,25-dihydroxyvitamin D3 (Catalog No. BML-DM300) was purchased from Enzo (Farmingdale, NY). T-PER tissue protein extraction reagent (Catalog No. 78510) was obtained from Thermo Scientific. All other reagents were from Sigma-Aldrich.

Animals

The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University. A total of intact male Sprague-Dawley rats aged 11 to 12 weeks (weighing approximately 300 g)

were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained in an AAALAC-accredited vivarium (Thomas Jefferson University) for 2 weeks before the start of the experiment. All rats were fed with standard laboratory diets (LabDiet 5001 Rodent Diet, Purina, St. Louis, MO) and food and water were provided ad libitum.

ABL Treatment in Rats

ABL (40 μ g/kg/day) or vehicle (sterile PBS) was administered subcutaneously daily for seven days. Blood was collected from fasted rats (~12 hours). Serum was prepared by allowing blood to clot at room temperature for 30 minutes before centrifugation at 1000g for 15 minutes. Plasma was prepared by collecting blood to EDTA pretreated tube on ice for 30 minutes before centrifugation at 1000g for 15 minutes. Serum and plasma were aliquoted for avoiding repeated freeze/thaw of samples and stored at -80 °C for biochemical analyses.

Bone Protein Extraction

Bone tissue collected from the distal femoral metaphysis in each group was weighed and cut into small pieces. Pre-cold T-PER tissue protein extraction reagent with protease inhibitor cocktail was added to bone tissue using a ratio of 1 g of bone tissue to 8 mL of T-PER reagent according to manufacturer's instructions. The samples were homogenized and then centrifuged with 10 000g for 5 minutes to pellet tissue debris. The supernatants were aliquoted and stored at -80 °C for analyses of Gla-Ocn, Glu-Ocn, and protein concentrations. Protein levels were measured by using Bradford dye and then calculated according to BSA standard curve.

Cell Culture

Rat osteosarcoma cells (osteoblast-like cells), ROS 17/2.8 cells (RRID: CVCL_0508), were cultured in DMEM/Ham's F-12 medium supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μ g/mL streptomycin (26, 27). The cells were maintained at 37 °C in a humidified atmosphere of 5% CO₂, 95% air. For 1,25-(OH)₂D formation and Ocn production, cells (2.5 \times 10⁵/well) were set up on a 12-well plate, and the assay was performed 12 to 24 hours later once the cells reached confluency. The cells were then incubated in the medium without fetal bovine serum in the presence or absence of ABL, 25(OH)D, 1,25-(OH)₂D, and vitamin K1. After 24 hours, the supernatants were collected for measurements of 1,25-(OH)₂D, Gla-Ocn, and Glu-Ocn formation, respectively.

Biochemical Measurements

1. Determination of Gla-Ocn and Glu-Ocn. The levels of Gla-Ocn and Glu-Ocn from rat serum (1:50 dilution) and supernatants from bone protein extraction (1:120 dilution) or cell culture supernatants (without dilution) in each group were quantified by using rat Gla-Ocn highly sensitive EIA kit and rat Glu-Ocn highly sensitive EIA kit, respectively, according to manufacturers' instructions. Gla-Ocn EIA kit uses a rat Ocn carboxy-terminus specific antibody as a capture antibody on a solid-phase plate. An enzyme-labeled antibody specific to Gla-Ocn (amino acids 4-30) is used as the detection antibody. Glu-Ocn EIA kit also uses a rat Ocn carboxy-terminus

specific antibody as a capture antibody on a solid-phase plate. A monoclonal antibody that is specific to the Glu residues that straddle positions 21 and 24 of Ocn is arranged as the detection antibody. Thus, these 2 kits can be used together for simultaneous detection of Gla-Ocn and Glu-ocn levels. A standard curve was plotted based on the results obtained from the standard solutions, and the curve was used to determine the corresponding concentrations of Gla-Ocn or Glu-Ocn based on the sample's absorbance. The concentrations of each sample were then multiplied by the dilution factor and expressed as a nanogram per milliliter in the blood or a nanogram per milligram of protein in the bone.

- Determination of 1,25-(OH)₂D. The rat plasma samples in each group were purified by immunoextraction (28). The amounts of 1,25-(OH)₂D from the purified plasma samples or cell culture supernatants were quantified using the EIA kit according to the manufacturer's instructions. A standard curve was plotted based on the results obtained from the standard solutions, and the curve was used to determine the corresponding concentrations of 1,25-(OH)₂D based on the sample's absorbance. The levels of blood 1,25-(OH)₂D were expressed as a picomole per liter. The levels of 1,25-(OH)₂D in cell supernatants were expressed as a picomole per gram of protein.
- Determination of 25-(OH)D. The rat serum samples in each group were quantified using the EIA kit according to the manufacturer's instructions. A standard curve was plotted based on the results obtained from the standard solutions, and the curve was used to determine the corresponding concentrations of 25-(OH)D based on the sample's absorbance. The levels of 25-(OH)D were expressed in nanomoles per liter.
- Determination of serum calcium: The calcium ion from rat serum was measured by a quantitative calcium colorimetric assay kit in accordance with the manufacturer's recommendations. A standard curve was plotted based on the results obtained from the standard solutions, and the curve was used to determine the corresponding concentrations of calcium ion based on the sample's absorbance.

cAMP Signaling Assay

cAMP measurements were performed in ROS 17/2.8 cells. The cells (1.25×10^5 /well) were set up to 24-well plate, and the assay was performed 12 to 24 hours later once the cells reached confluency. The cells were then incubated in the medium without fetal bovine serum. The cells were treated with vehicle or ABL (for 15 minutes) in the pretreatment of phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (1 mM) for 15 minutes. cAMP formation was detected using the cAMP direct EIA kit. The samples were analyzed by using a cAMP standard curve (25, 29).

Statistical Analysis

All statistical analyses were performed using GraphPad Prism, version 9. Results in the figures are presented as bar graphs with dot plots, which maximize visualization of the data distribution. For the animal study, n indicates the rat number, and each sample was measured twice to get the average value. For the cell study, n indicates the number of independent experiments with triplicated wells in each group. Two-tailed

Student *t*-test was performed to determine the treatment effects on rats between 2 groups. The significance level for the ratio of Gla-Ocn to Glu-Ocn was adjusted by using Bonferroni method, which multiplies the *P* value by 2. Multiple comparisons for cell studies were evaluated by 1-way ANOVA followed by Bonferroni posttest. Correlations between 1,25-(OH)₂D and Ocn formation were analyzed by Pearson coefficient of correlation. *P* < .05 was considered significant.

Results

ABL Increases Ocn and 1,25-(OH)₂D Formation Without Raising Blood Calcium Levels

ABL (25 µg/kg) daily injection was reported to significantly increase blood total Ocn levels in rats even as early as 1 week (5). We explored if ABL (40 µg/kg) daily injection for 7 days affected Gla-Ocn and Glu-Ocn levels and their ratio in rats. ABL significantly increased Gla-Ocn levels compared with vehicle control (Fig. 1A). However, Glu-Ocn value increase was less robust than Gla-Ocn, although it was significantly increased (Fig. 1B). The ratio of Gla-Ocn to Glu-Ocn in ABL group was significantly higher than that in vehicle control (Fig. 1C). In addition, ABL significantly augmented 1,25-(OH)₂D levels compared with vehicle control (Fig. 1D). However, we did not find that ABL increased serum blood calcium levels (Fig. 1E). Furthermore, we found that ABL slightly reduced the 25-(OH)D levels compared with vehicle control (Fig. 1F), which is different from the data that ABL treatment significantly increased the blood 1,25-(OH)₂D levels. Vitamin D can be metabolized into other forms of metabolites that circulate in the blood. These metabolites include 25-(OH)D and 24,25-dihydroxyvitamin D [24,25-(OH)₂D]. The data in Fig. 1F showed that about 50 nmol/L of 25-(OH)D existed in the blood, which may be converted to 24,25-(OH)₂D in the presence of 24-hydroxylase. To test the specificity of the 1,25-(OH)₂D assay, samples that contain 25-(OH)D (50 nmol/L) and 24,25-(OH)₂D (50 nmol/L) were measured with a 1,25-(OH)₂D EIA kit. We found that the 1,25-(OH)₂D EIA kit was incapable of detecting the 25-(OH)D and 24,25-(OH)₂D amounts (10).

To gain further insight into the effect of ABL on Ocn deposition to the bone matrix, we extracted bone proteins in femoral tissue and measured the levels of Gla-Ocn and Glu-Ocn in bone. We found ABL significantly increased the Gla-Ocn levels in bone (Fig. 1G). ABL also significantly increased the Glu-Ocn amount in bone, but the magnitude is much less than that of Gla-Ocn (Fig. 1H). In addition, the ratio of Gla-Ocn to Glu-Ocn in ABL group was notably higher than in control group (Fig. 1I).

Blood Gla-Ocn But Not Glu-Ocn Correlates With 1,25-(OH)₂D in Rats Treated With ABL

In human and rat osteoblastic cells, there is a vitamin D response element sequence in the promoter region of Ocn gene, which is in response to 1,25-(OH)₂D through binding the vitamin D receptor (30, 31). Multiple lines of evidence were presented that 1,25-(OH)₂D administration to humans and rats or addition of 1,25-(OH)₂D to osteoblasts induced total Ocn formation (32-34). We did the correlations of blood 1,25-(OH)₂D with Gla-Ocn and Glu-Ocn, and their ratio of Gla-Ocn to Glu-Ocn with or without ABL treatment. As expected, no significant correlation was found between 1,25-(OH)₂D with Gla-Ocn or Glu-Ocn in

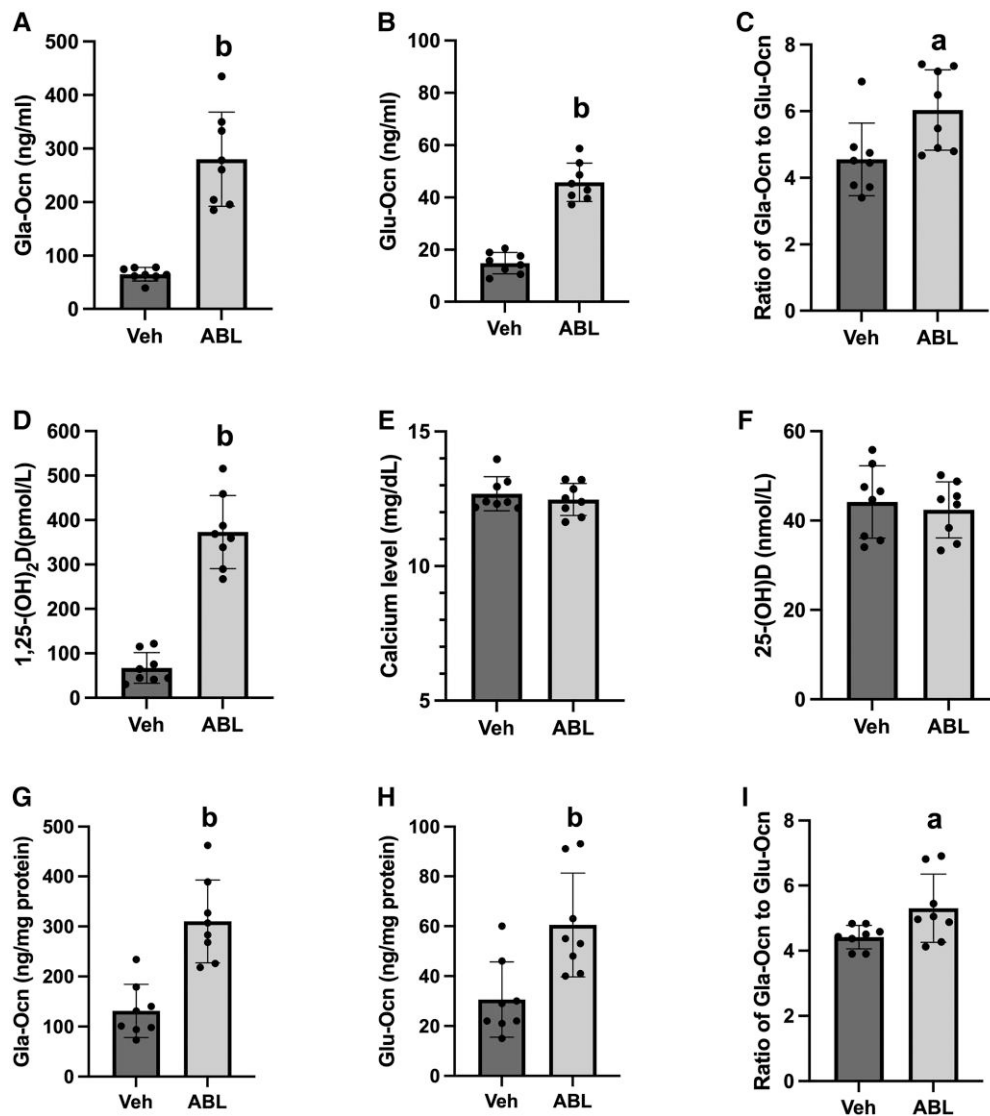


Figure 1. ABL increases Gla-Ocn and 1,25-(OH)₂D formation without raising blood calcium levels. (A) ABL significantly increases blood Gla-Ocn levels compared with vehicle (Veh) control. (B) ABL significantly increases blood Glu-Ocn levels, but the magnitude is much less than that of Gla-Ocn. (C) ABL significantly increases the ratio of blood Gla-Ocn to Glu-Ocn. (D) ABL significantly augments 1,25-(OH)₂D levels. (E) ABL does not increase blood calcium levels. (F) ABL does not increase blood 25-(OH)D levels. (G) ABL significantly increases Gla-Ocn levels in the bone. (H) ABL significantly increases Glu-Ocn levels in the bone, but the magnitude increase is much less than that of Gla-Ocn. (I) ABL significantly increases the ratio of Gla-Ocn to Glu-Ocn in the bone. $n = 8$. ^a $P < .05$, ^b $P < .01$, compared with Veh control.

rats treated with vehicle (Fig. 2A, 2B, and 2C). However, there was a significantly positive correlation between 1,25-(OH)₂D and Gla-Ocn (Fig. 2D), or the ratio of Gla-Ocn to Glu-Ocn (Fig. 2F) in rats treated with ABL. In addition, no significant positive correlation was found between 1,25-(OH)₂D and Glu-Ocn (Fig. 2E), which is possibly because Glu-Ocn value increase was less robust than Gla-Ocn after ABL treatment.

ABL Is Able to Stimulate 1,25-(OH)₂D Formation in Nonrenal Cells

Renal proximal tubular cells are the primary site for inducing 1,25-(OH)₂D formation in the presence of 1 α -hydroxylase (18). In addition, nonrenal cells, such as human primary osteoblasts and osteosarcoma cell line (24), also express 1 α -hydroxylase and synthesize 1,25-(OH)₂D. It is known that PTH-stimulated

1,25-(OH)₂D formation occurs through the activation of PTHR, which is via the Gs/cAMP pathway (35, 36). As with PTH itself, ABL can also activate PTHR. We tested whether ABL could stimulate 1,25-(OH)₂D formation in ROS17/2.8 cells. The data in Fig. 3A showed that ABL concentration-dependently increased cAMP formation, indicating ABL can activate PTHR in these cells. As expected, ABL failed to induce 1,25-(OH)₂D formation without adding 25-(OH)D, the substrate of 1 α -hydroxylase. However, after addition of 25-(OH)D to the culture medium, 25-(OH)D was concentration-dependently converted to 1,25-(OH)₂D (Fig. 3B). Importantly, ABL was able to stimulate 1,25-(OH)₂D formation, which also occurred in a 25(OH)D concentration-dependent manner (Fig. 3B). Furthermore, the 1,25-(OH)₂D EIA kit only detected 1,25-(OH)₂D levels (Fig. 3B, left) but failed to detect 25-(OH)D amounts (Fig. 3B, right), further confirming the 1,25-(OH)₂D EIA kit is highly specific and sensitive.

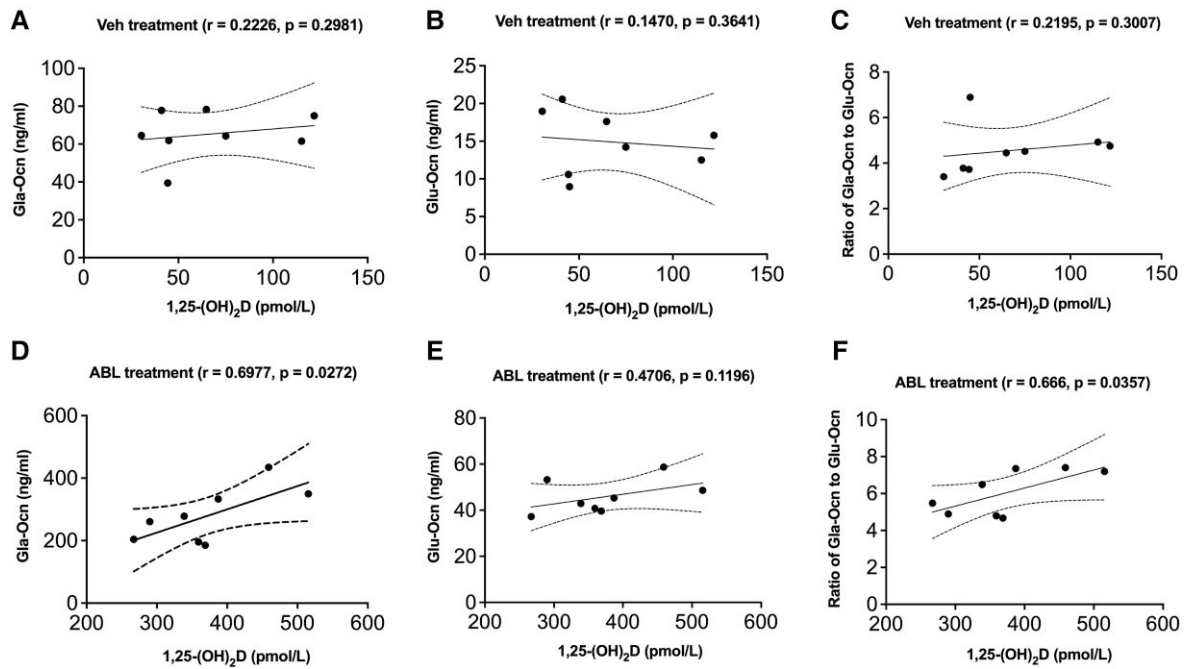


Figure 2. Blood Gla-Ocn but not Glu-Ocn levels positively correlates with 1,25-(OH)₂D amounts in rats treated with ABL. Rats were treated with ABL for 7 days. The levels of Gla-Ocn, Glu-Ocn, and 1,25-(OH)₂D were detected as described in Material and Methods. Results of Pearson coefficient of correlation test were conducted for 1,25-(OH)₂D with Gla-Ocn, Glu-Ocn, and the ratio of Gla-Ocn to Glu-Ocn in rats treated with vehicle (A, B, C) or ABL (D, E, F). Solid lines represent regressions and dot lines represent 95% CIs.

ABL Has Less Direct Effect on Ocn Formation

Although ABL treatment can increase Ocn formation in rats, it is unknown whether the effect of ABL on Ocn formation is direct or indirect. A previous report indicated that 1,25-(OH)₂D could induce total Ocn formation, but the effect of 1,25-(OH)₂D on Gla-Ocn and Glu-Ocn production is unknown. Because the majority of Ocn is carboxylated in posttranslational modification in osteoblasts, we hypothesized that 1,25-(OH)₂D mostly increased Gla-Ocn formation. Data in Fig. 4A showed that 1,25-(OH)₂D induced Gla-Ocn formation in ROS17/2.8 cells in an 1,25-(OH)₂D concentration-dependent manner, but also increased small amount of Glu-Ocn production. It was established that vitamin K is a cofactor of γ -glutamyl carboxylase for posttranslational carboxylation of Ocn (37). After different concentrations of vitamin K1, the main form of vitamin K, were added to the culture medium, Gla-Ocn formation was increased in vitamin K1-dependent manner, whereas the amount of Glu-Ocn was also decreased in vitamin K1-dependent fashion (Fig. 4B). ABL alone had little effect on both Gla-Ocn and Glu-Ocn formation (Fig. 4C), which is consistent with previous report that PTH failed to stimulate total Ocn formation in cultured fetal rat calvariae (34). Thus, these results suggest that the effect of Ocn formation induced by ABL in rats is indirect.

ABL Acts Synergistically With 1,25-(OH)₂D to Stimulate Gla-Ocn Formation

The Gla-Ocn formation was significantly increased, when ABL was combined with 1,25-(OH)₂D (Fig. 5A). To further validate the finding, the cell culture was added ABL with different concentrations of 25-(OH)D, which is a biologically inactive form of vitamin D. We found that 25-(OH)D concentration-dependently increased Gla-Ocn synthesis, indicating that 25-(OH)D was converted to 1,25-(OH)₂D in

ROS17/2.8 cells (Fig. 5B). ABL further promoted the Gla-Ocn formation in the presence of 25-(OH)D (Fig. 5B). Collectively, ABL collaborates with 1,25-(OH)₂D to stimulate Gla-Ocn production and Gla-Ocn is the downstream signaling of 1,25-(OH)₂D.

Discussion

The effect of ABL on PTHR signaling has not been extensively studied compared with PTH(1-34). It is established that PTH(1-34) in vivo can enhance 1,25-(OH)₂D formation and Ocn secretion, respectively (38, 39). However, the effects of ABL on Glu-Ocn and Gla-Ocn secretion have not been explored yet. We propose that the effect of ABL on Gla-Ocn/Glu-Ocn formation is similar to that of PTH(1-34). The Glu-Ocn, which is released from the bone matrix to the circulation when bone resorption occurs, has been considered as a bone-derived endocrine hormone with manifold functions including glucose metabolism and male fertility (12, 40). In 2020, 2 laboratories independently found that new Ocn knockout mice exhibit normal bone mineral density though they display a crystal misalignment along the collagen fibrils consistent with a low degree of crystal maturation and increased brittleness (12, 40). These data are consistent with findings from Ocn knockout rats (30) but are inconsistent with data from the original Ocn knockout mice (41). Similar to human Ocn gene (*Bglap*), the rat Ocn gene locus consists of a single copy of Ocn. 1,25-(OH)₂D binding to vitamin D receptor can affect Ocn formation because there is a vitamin D response element sequence found in the promoter region of Ocn gene (30, 31). The transcription of the rat Ocn gene, as with the human Ocn gene, is upregulated by 1,25-(OH)₂D, whereas the mouse Ocn genes are downregulated by vitamin D (30, 31). Thus, we chose the rat model and rat cell line to study the ABL effects on Ocn and 1,25-(OH)₂D formation;

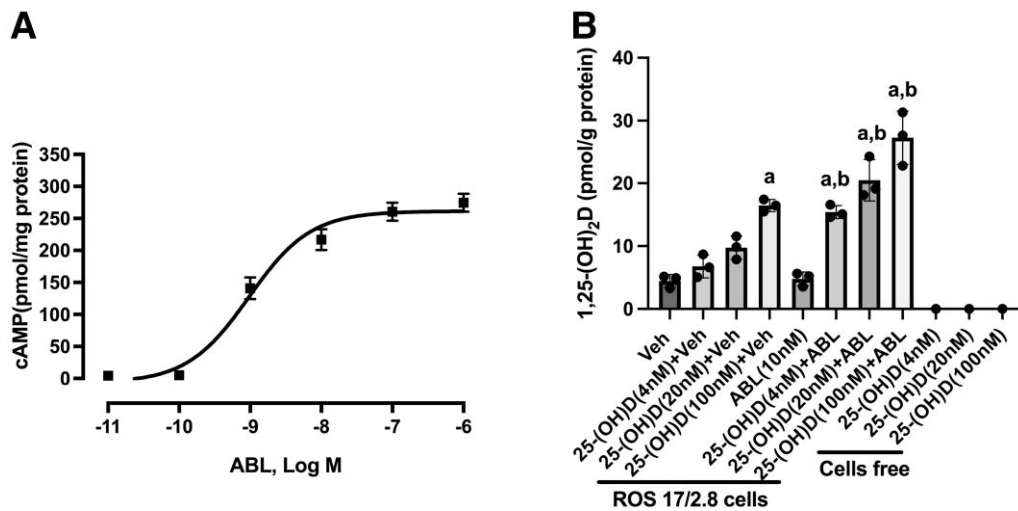


Figure 3. ABL is able to stimulate 1,25-(OH)₂D formation in nonrenal cells. (A) ABL is concentration-dependent increase of cAMP formation. (B) ABL stimulates 1,25-(OH)₂D formation in the presence of 25-(OH)D. *n* = 3. ^a*P* < .01 compared with Veh; ^b*P* < .01 compared with ABL.

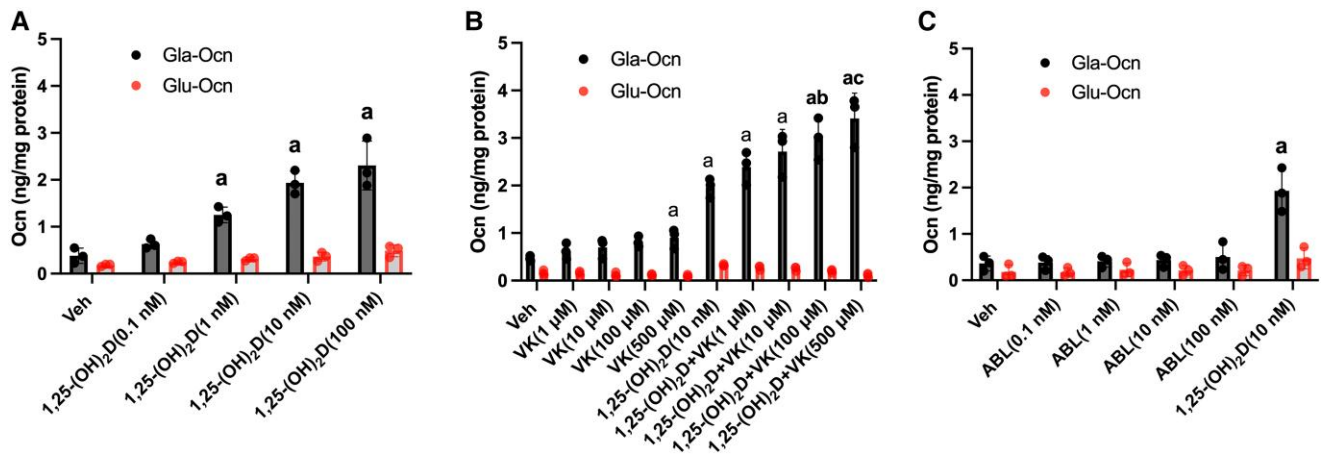


Figure 4. ABL has less direct effect on Ocn formation. (A) 1,25-(OH)₂D induces Gla-Ocn formation in concentration-dependent manner. (B) Vitamin K1 (VK) enhances 1,25-(OH)₂D-induced Gla-Ocn formation but reduces 1,25-(OH)₂D-induced Glu-Ocn production. (C) ABL has little effect on Gla-Ocn and Glu-Ocn formation. *n* = 3. ^a*P* < .01, compared with Veh; ^b*P* < .05, ^c*P* < .01, compared with each concentration of VK.

the data we found in the current study may properly be translated into a human study.

In the present study, ABL has little effect on increasing Gla-Ocn formation but significantly increases Gla-Ocn synthesis when adding 1,25-(OH)₂D or 25-(OH)D to ROS17/2.8 cells, suggesting Gla-Ocn formation in rats induced by ABL is mediated by 1,25-(OH)₂D. These data also confirm there is a crosstalk between the PTHR and vitamin D receptor (42, 43). Importantly, our data showed that ABL significantly increased blood levels of 1,25-(OH)₂D and Gla-Ocn but maintain the normal blood calcium levels. The concentration of free calcium in plasma is set by the extracellular action of noncollagenous proteins and hydroxyapatite (44). Ocn is one of such noncollagenous proteins and plays an essential role in calcium homeostasis (8, 44). Thus, the seemingly paradoxical effects of ABL on 1,25-(OH)₂D formation and blood calcium level change may be attributed to the feature of 2 forms of Ocn for binding ionized calcium. Gla-Ocn exhibits high affinity by binding to Ca²⁺, whereas Glu-Ocn has no

affinity to Ca²⁺ (11, 12). Gla-Ocn is synthesized by osteoblasts and released into the bone matrix and binds to hydroxyapatite at bone surfaces. We propose that the increased Gla-Ocn after ABL treatment will elevate the saturation point for the solubility of hydroxyapatite, thereby permitting calcium ions in the extracellular fluid at bone sites to move to the bone mineral surface (44). This nonhormonal regulation of calcium levels may explain why ABL has anabolic effects without increasing blood calcium levels. However, we do not exclude the hormonal regulation of calcium homeostasis (42, 43). Thus, there are some limitations of this study in that we did not measure the blood endogenous PTH and calcitonin levels during the ABL treatment. Furthermore, we only examined the biochemical markers at the endpoint of the study. Development of better assay systems that can measure the levels of the blood calcium, Gla-Ocn, and Glu-Ocn in smaller blood samples from live rats at earlier time points will be helpful for future studies. Bones are not inert structures but undergo constant remodeling. When osteoclasts are activated to form the acidic condition,

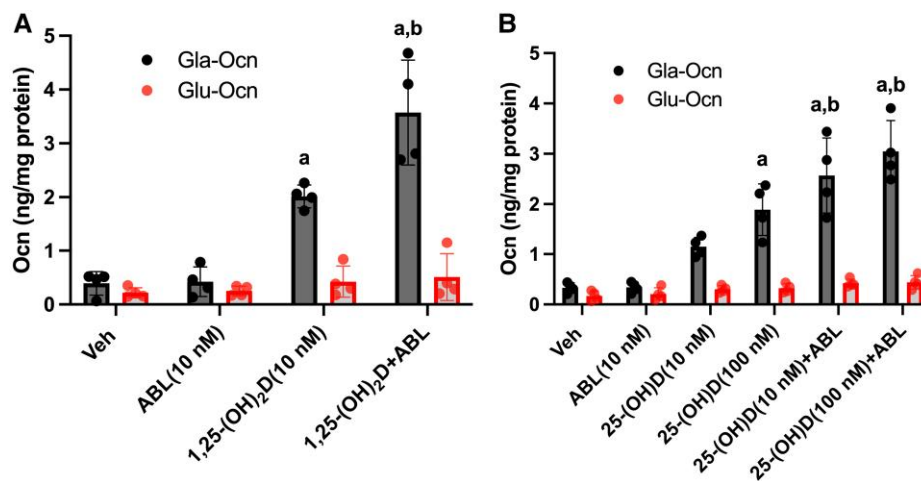


Figure 5. ABL acts synergistically with 1,25-(OH)₂D to stimulate Ocn production. (A) ABL promotes 1,25-(OH)₂D-induced Gla-Ocn formation. (B) ABL stimulates Gla-Ocn formation in the presence of 25-(OH)D. n = 4. ^aP < .01, compared with Veh; ^bP < .01, compared with 1,25-(OH)₂D or 25-(OH)D.

Gla-Ocn in the bone matrix can be converted to Glu-Ocn, which is then released into the circulation. Our data showed that Gla-Ocn level was significantly increased compared with Glu-Ocn value in the blood and bone after ABL treatment, indicating ABL skews the balance of bone remodeling to favor bone formation. Because of a short period of ABL treatment, the relationship between Ocn change and bone mass was not investigated, which is one of the limitations in the current study.

Vitamin D can be metabolized into other forms, including 24,25-(OH)₂D and 1,24,25-trihydroxyvitamin D. In contrast to 1,25-(OH)₂D, the same concentrations of 24,25-(OH)₂D did not stimulate total Ocn synthesis in the cultured fetal rat calvariae (34). However, 24,25-(OH)₂D in higher concentrations also stimulated total Ocn formation in human osteoblastic cells (24). In addition, 1,24,25-trihydroxyvitamin D was reported to stimulate total Ocn synthesis and its ability on Ocn expression was equipotent to 1,25-(OH)₂D in human osteoblastic cells (24). Although it has been demonstrated that osteoblasts have 1 α -hydroxylase and 24-hydroxylase activities, the 24-hydroxylase capacity is lower than that of 1 α -hydroxylase (24, 45). Whether ABL has effects on 24-hydroxylated metabolites-induced Ocn synthesis needs to be explored in the future study. The 1,25-(OH)₂D EIA kit that is a complete assay system for the purification of 1,25-(OH)₂D in plasma samples by immunoextraction with specific monoclonal 1,25-(OH)₂D antibody followed by quantitation of EIA assay that demonstrated there are no cross-reactivities with 24,25-(OH)₂D. However, we did not confirm the immunoextraction purity of 1,25-(OH)₂D by mass spectrometry to rule out the possible contamination by 24-(OH)D species, which is another limitation of the current study.

In summary, the signaling pathway of ABL/1,25-(OH)₂D/Gla-Ocn may provide insight into understanding the mechanism of anabolic effects of ABL for the treatment of osteoporosis. Our data suggest that (1) blood levels of Gla-Ocn and Glu-Ocn or the ratio of Gla-Ocn to Glu-Ocn can be implicated as bone biomarkers to evaluate drug efficacy for simultaneous monitoring of bone formation and resorption; and (2) 1,25-(OH)₂D may act preferentially in promoting bone mineralization through Gla-Ocn for mediating calcium association with hydroxyapatite rather than bone loss through calcium mobilization. Further study for administration of a prolonged period of ABL treatment or using Ocn knockout

rats may be required to confirm this signaling pathway for the contribution of underlying anabolic mechanism of ABL and other anabolic agents on bone.

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Disclosures

The authors have nothing to disclose.

Data Availability

Some or all datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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